Preservation of rhizobia by lyophilization with trehalose[1]

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Abstract – The objective of this study was to determine if trehalose can effectively enhance the viability and storage lifetime of *Rhizobium tropici* and *Rhizobium etli* after lyophilization as compared to the traditional protective combination of sucrose and peptone. Two strains of bean-nodulating rhizobia were effectively preserved in the lyophilized state by 100 mM trehalose, and survived for at least 12 days, even when stored under non-ideal conditions. Trehalose provided better protection than the sucrose/peptone combination. When protective excipients were introduced inside the cells, strain CFN 42 was more sensitive to the type of additive used than strain CIAT 899, probably because CIAT 899 produces β (1-2) glucan, which may have a protective effect. β (1-2) glucan combined with sucrose protected better than trehalose against leakage from freeze-dried liposomes, but by itself it had no protective capability. There was a high level of unfrozen water associated with the hydrated glucan, in addition to a thermal event at approximately 70ºC, corresponding to an observed gel-sol transition in the glucan. These findings may shed light on the mechanism by which the glucan contributes to the protection of dried cells and liposomes, and may lead to further improvements in rhizobial inoculants.

Index terms: storage, inoculation, symbiosis, glucan.

Preservação de rizóbio por liofilização com trealose

Resumo – O objetivo deste estudo foi determinar se a trealose pode aumentar a viabilidade e o tempo de preservação de estirpes de *Rhizobium tropici* e *Rhizobium etli* após a liofilização, comparada com a proteção proporcionada pela combinação tradicional de sacarose e peptona. Duas estirpes de rizóbio para feijão foram preservadas liofilizadas com 100 mM de trealose e sobreviveram por pelo menos 12 dias, mesmo quando armazenadas sob condições não-ideais. A trealose proporcionou uma proteção superior à combinação de sacarose peptona. Quando agentes protetores foram introduzidos dentro da célula, a estirpe CFN 42 foi mais sensível em relação ao tipo de agente usado do que a estirpe CIAT 899, provavelmente porque a estirpe CIAT 899 produz β (1-2) glucano, que parece ter também um efeito protetor. β (1-2) glucano combinado com sacarose protege mais os lipossomas liofilizados de perdas do que a trealose. Entretanto, o β (1-2) glucano, isoladamente, não tem capacidade de proteção. Foi observado um alto nível de água descongelada associado com o glucano hidratado; além disso, o evento termal foi de, aproximadamente, 70ºC, o que correspondeu à transição gel-sol do glucano. Essas descobertas são importantes para o entendimento dos mecanismos com que o glucano contribui para a proteção de células e lipossomas liofilizados e para aumentar a sobrevivência do rizóbio nos inoculantes.

Termos para indexação: armazenamento, inoculação, simbiose, glucano.

Introduction

Symbiotic nitrogen fixation results from the interaction of a legume host with rhizobia, leading to root nodulation of the host plant. As there are several native species of *Rhizobium* present in the soil, introduced strains must be present in extremely large numbers in order to compete with native rhizobia and have agricultural significance. Thus, one of the

main problems of the inoculation industry is to keep rhizobial cells viable in large numbers in the inoculant. This is especially important with the recent tendency to shift from peat-based to alternative inoculants containing freeze-dried rhizobia.

Although certain rhizobial strains have been preserved for long-term storage by lyophilization (Somasegaran & Hoben, 1994), this procedure can cause serious problems, such as the denaturation of sensitive proteins (Hellman & Cammack, 1983; Carpenter et al., 1987), and cell death for some organisms (Mazur, 1968; MacKensie, 1977). Several types of excipients, such as sugars and other carbohydrates, have been used to alleviate the damage caused by freeze drying (Simione & Brown, 1991). Recently, the disaccharide trehalose, which accumulates to extremely high levels in several dehydration-tolerant yeast strains, has been used with great success to protect bacteria from lyophilization-induced damage (Israeli et al., 1993; Leslie et al., 1995). *Escherichia coli* cells freeze dried in 100 mM trehalose showed as high as 75% survival after rehydration, compared to less than 10% survival after freeze drying in the absence of added sugar (Leslie et al., 1995).

Based on what is known about stabilization of biomolecules by trehalose *in vitro* (Carpenter et al., 1987; Crowe et al., 1987; Tsvetkov et al., 1989; Harrigan et al., 1990; Crowe et al., 1996), the protection against drying afforded to the bacteria by trehalose is likely to be due to its effects on proteins and membranes.

Equally important to bacterial survival is the maintenance of cell membranes in the liquid crystalline phase at critical periods during drying and rehydration. When lipid bilayers are dried in the absence of carbohydrates, the gel to liquid crystalline phase transition temperature is elevated by as much as 50-80°C, thus the membranes will pass through a phase transition as they are rehydrated (Crowe & Crowe, 1988).

Most carbohydrates can form glasses, and this vitrification is also an important characteristic of trehalose in regard to its protection of bacterial cells during lyophilization. Indeed, vitrification has been shown to be necessary but not sufficient for the preservation of liposomes during freeze drying (Crowe et al., 1994). The glass transition temperature (T_g) of trehalose is higher than that of sucrose, enabling it

to remain vitrified at higher temperatures than sucrose (Crowe et al., 1994; Oliver et al., 1996). This is the likely explanation for the finding that trehalose protects membranes and intact cells better than sucrose during and after drying, although the two disaccharides are similar in most other aspects (Crowe et al., 1996).

Two rhizobial species which establish an effective symbiosis with the common bean (*Phaseolus vulgaris* L.) were redefined as *Rhizobium tropici* (Martinez-Romero et al., 1991) and *Rhizobium etli* (Segovia et al., 1993).

The objective of this study was to determine if trehalose can effectively enhance the viability and storage lifetime of *Rhizobium tropici* and *R. etli* after lyophilization, as compared to the traditional protective combination of sucrose and peptone.

Material and Methods

Rhizobium tropici, strain CIAT 899, and *Rhizobium etli*, strain CFN 42, were grown in YEM broth with shaking at 28°C for 72 hours. The number of cells was quantified, and values of 3.2×10^{11} and 2.0×10^{11} per mL were obtained for strains CIAT 899 and CFN 42, respectively. Cell suspensions were centrifuged in Eppendorf tubes and cell pellets were resuspended in a solution of 100 mM trehalose or 7% peptone (w/v) containing 7% (w/v) sucrose. In order to isolate the β (1-2) glucan produced by the CIAT 899 strain, colonies were grown on YEM medium plates at 28°C for seven days. β (1-2) glucan was extracted by scratching the plates with a spatula to remove the bacterial colonies, and suspending them in sterile water. The dissolved β (1-2) glucan was separated from the bacterial cellular residue by filtering through an 0.2 μm pore Millipore filter, freeze dried, and quantitated.

A Perkin Elmer Fourier transform infrared (FTIR) spectrometer 1750 optical bench was used to study the gel to liquid crystalline phase transition temperature (T_m) of the bacterial membranes, as previously described (Crowe et al., 1989). Briefly, samples were sandwiched between two CaF_2 windows and loaded onto a temperature-controlled holder. Temperature of the samples was regulated with a Peltier device designed by Paige Electronics (Davis, CA). Samples were scanned in the range of 3,000-1,000 cm^{-1} and examined in the range of 2,900-2,800 cm^{-1}, for the symmetric CH_2 stretch, which shifts by approximately 3 cm^{-1} as the lipid is chilled through its phase transition.

Trehalose and other excipients were introduced into the bacterial cells by passing the cells through their gel to

liquid crystalline phase transition temperature in the presence of a relatively high external solute concentration (100 mM trehalose, or sucrose + peptone, both at 7% w/v). Under these conditions, solute will pass into the cells and reduce its concentration gradient, as the cell membranes become transiently leaky (Leslie et al., 1995; Beattie et al., 1997). Cell suspensions (500 µL) were placed into 1.5 mL Eppendorf tubes and capped (samples used in lyophilization studies were capped with perforated caps). The tubes were placed in racks inside a Cryomed Regulated Cooling System 910 with a Model 1010 Microcomputer (Custom Biogenics Systems, Shelby Township, MI) and chilled through the bacterial cell membrane phase transition (from 22°C to -5°C) at a rate of 5°C/min.

Once below T_m, cell suspensions were chilled with the Cryomed freezer at a rate of 99°C/min to a temperature of -80°C. Frozen cells were transferred to a Virtis Freezemobile 25SL freeze dryer (Gardiner, NY), which has a condenser temperature of -50°C and a vacuum of 10 mTorr. Lyophilization was allowed to continue for at least 18 hours, at which point residual water content was about 0.02 g water/g dry weight.

The freeze-dried cells were rehydrated at room temperature (22°C) with sterile water in a tissue culture hood, prior to plating on YEM broth medium for colony counts. Alternatively, the Eppendorf tubes containing the freeze dried cells were placed into incubators at 4, 30, or 40°C and partially rehydrated during storage in a sealed chamber over a saturated NaBr solution (58% relative humidity at 22°C) (Weast, 1984). After 280 hours of incubation, three samples were taken from each temperature to determine cell survival. The number of colonies was analyzed after log transformation in a completely randomized design.

Egg phosphatidylcholine (EPC, from Avanti Polar Lipids, Alabaster, AL) large unilamellar vesicles (LUVs) were prepared by extrusion in the presence of 100 mM carboxyfluorescein (CF) as previously described. External CF was removed by passing the liposome sample over a Sephadex G 50-80 column (1x20 cm). Trehalose, sucrose, or β (1-2) glucan was added to the liposome suspension at a concentration of 2 g/g lipid. Liposome samples (50 µL) were placed into Eppendorf tubes with perforated caps, frozen in liquid nitrogen, and transferred directly to the Virtis lyophilizer for at least 18 hours. After lyophilization and rehydration with water, a small volume of LUVs (5 µL) was transferred to a cuvette containing 3 mL water. Samples were excited at 460 nm, and emission was measured at 550 nm on a Perkin Elmer LS-5 fluorescence spectrophotometer. Intravesicular CF concentrations are self-quenching, so the emission at 550 nm was due only to

external CF. Percent leakage was calculated after adding 50 µL of 1% Triton X-100 to each sample to cause total (100%) leakage. Percent retention was calculated as the difference between 100 and the measured percent leakage.

The glass transition temperature (T_g) of the carbohydrates at different levels of hydration was measured with a high sensitivity differential scanning calorimeter (DSC-7) from Perkin-Elmer, cooled by a liquid nitrogen reservoir. Carbohydrate samples (5 mg approximately) were dried using small aluminum pans in a dry box maintained at 0% relative humidity (RH) for several days and stored overnight under vacuum. The pans were either pressure sealed inside the dry box or transferred to RH chambers, maintained at specific relative humidities over saturated salt solutions, for 24 hours before sealing (Weast, 1984). The DSC-7 head was flushed with He, and the box with dry N_2. Carbohydrate samples were scanned between -180°C and 200°C at 20-50°C/minute. Sample pans were weighed on a Cahn C-33 electrobalance before and after DSC scanning to ensure that no water was lost during the procedure. Sample pans were punctured and incubated at 120°C for at least 24 hours and re-weighed. This incubation and re-weighing step was repeated until sample weight remained constant for two consecutive days, allowing accurate calculation of sample hydration during the DSC scan.

The unfrozen water fraction was calculated from DSC scans of frozen hydrated glucan samples. The enthalpy of ice melting in the frozen glucan samples was calculated by integrating the area under the ice-melting endotherm. Since the enthalpy of ice melting is known to be 333.88 J/g (Weast, 1984), the percentage of total water present in the form of ice can be estimated by dividing the experimentally obtained enthalpy of ice melting by 333.88. The remainder is the amount of water present in the unfrozen state, which is reported as g water/g carbohydrate dry weight.

Results and Discussion

FTIR spectroscopy was used to determine the gel to liquid crystalline phase transition temperatures of the membranes of rhizobial strains CIAT 899 and CFN 42. The mid-point of the transition (T_m) was centered at 2°C for CFN 42 (Figure 1). Similar results were obtained for strain CIAT 899 (0°C). This is not unusual, as *Escherichia coli* also has a T_m of 0°C. However, variability in membrane composition among bacterial species can lead to differences in T_m, as evidenced by *Bacillus thuringiensis*, which has a T_m centered on 12°C (Leslie et al., 1995).

When rhizobial cells were freeze-dried in the absence of added sugar, the phase transition temperature was elevated to 47°C (Figure 1). If trehalose (at 2 g/g dry weight) was included before freeze-drying, however, the T_m remained low (9°C), an effect that protects the membrane from passing through a phase transition upon rehydration (Harrigan et al., 1990).

Knowledge of the membrane T_m allowed introduction of protective additives to the cytosol of the bacterial cells. In the presence of 100 mM trehalose, cell suspensions were chilled slowly through their phase transition (5°C/min from 20 to -5°C). This procedure leads to an equilibration of excipient across cell membranes as shown previously by using ^{14}C trehalose [10,28]. This procedure was used to load rhizobial cells with either 100 mM trehalose or the combination of sucrose and peptone, both at 7% (w/v).

Trehalose was compared with the combination of sucrose and peptone for its ability to preserve rhizobia cells during lyophilization. Separate suspensions of CIAT 899 and CFN 42 cells were freeze-dried in the presence of either trehalose or sucrose and peptone. CFN 42 cells were more sensitive than CIAT 899 cells to the type of excipient used (Figure 2). CFN 42
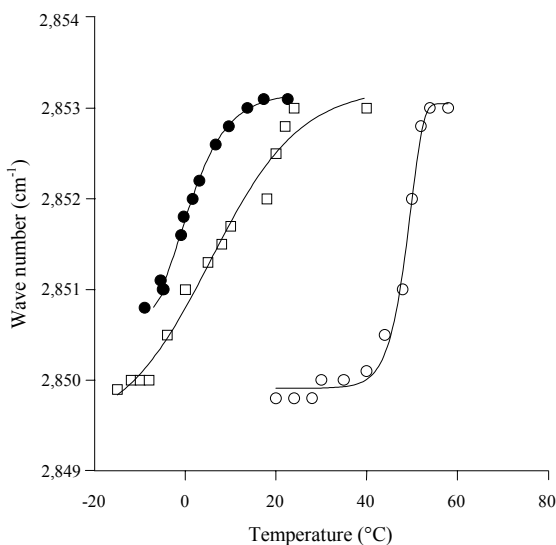
cells showed a significant decrease in survival when they were lyophilized in the sucrose/peptone combination but this was not evident when the cells were lyophilized with trehalose. CIAT 899 cells, in contrast, were preserved by either additive.

CIAT 899 and CFN 42 cells lyophilized with either sucrose/peptone or trehalose were stored for 280 hours at temperatures of 4, 30, or 40°C in a sealed chamber over a saturated NaBr solution. In all cases, percent survival decreased as the storage temperature increased (Figure 3). Trehalose provided a greater degree of protection to the lyophilized cells than the sucrose/peptone combination, as survival was higher in all cases when the cells were freeze-dried in the presence of trehalose. CIAT 899 cells were much less sensitive to the type of excipient used than CFN 42 cells. Similar to the case of survival immediately following lyophilization. This is clear from the results that there was only a slight difference in survival between CIAT 899 cells preserved in trehalose versus sucrose/peptone. In con-
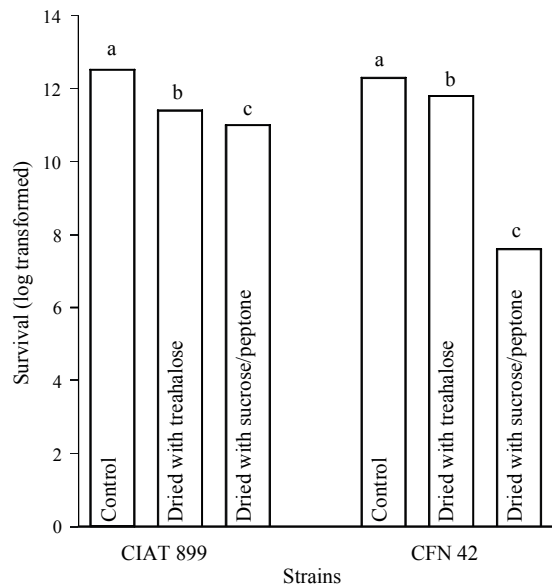

Figure 1. Gel to liquid crystalline phase transitions (T_ms) in rhizobial cells (strain CFN 42), as measured by FTIR. T_m for cells dried without added sugar (○) was 47°C, whereas T_ms for hydrated cells (●) and cells dried with trehalose (□), were 2°C and 9°C, respectively.

Figure 2. Effect of freeze-drying on the cell survival (log of viable cells/mL) of two *Rhizobium* strains treated with either 100 mM sucrose + 7% (w/v) peptone or 100 mM trehalose, as compared to the non-lyophilized control grown in YEM medium. Mean separations for each rhizobia strains by LSD at 5% probability level.

trast, there was a significant difference between the survival of CFN 42 cells depending upon whether they were preserved with trehalose or the sucrose/peptone combination, especially at higher temperatures.

The disparity between CIAT 899 and CFN 42 in sensitivity to preservative may be partly explained by earlier evidence indicating *Rhizobium tropici* is relatively resistant to environmental stresses, such as high temperature and field acidity (Hungria et al., 1993; Graham et al., 1994). One explanation may be that this bacterial strain produces a large amount of β (1-2) glucan, which has been suggested to have its own protective capability (Hungria et al., 1993; Graham et al., 1994).

In order to examine the role of the secreted β (1-2) glucan in the protection of the lyophilized CIAT 899 cells, several carbohydrates were tested, alone and in combination, for their effectiveness in protecting phospholipid vesicles from leaking after lyophilization, storage, and rehydration.

Trehalose was much more effective than sucrose in protecting the freeze-dried liposomes during storage under these conditions (Figure 4). Also, for both trehalose and sucrose, the liposomes leaked more carboxyfluorescein when incubated at the higher
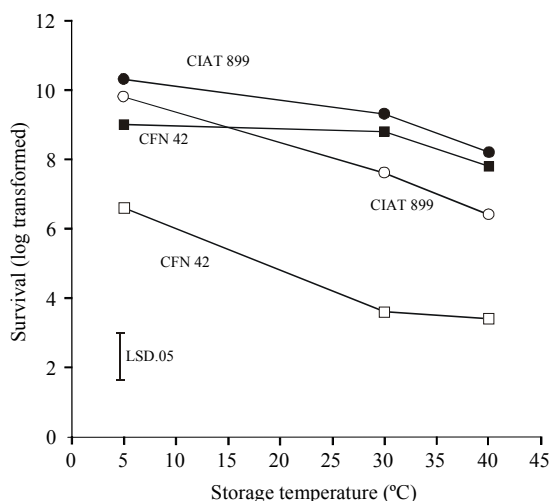
temperature. Both these effects are possible due to the relationship between carbohydrate vitrification and liposome preservation. Once the protective carbohydrate is no longer in the vitrified state ($T > T_g$), the liposomes fuse and leak their contents. This explains why the samples stored at the highest temperature showed lower retention, and also why the liposomes preserved with trehalose had higher retention than those preserved with sucrose (T_g for trehalose $>> T_g$ for sucrose) (Crowe et al., 1996). Furthermore, the T_g for a given carbohydrate glass drops dramatically as water is added (Roos & Karel, 1991), which helps to explain the precipitous drop in retention as the liposome samples adsorbed water.

The most striking result however, is that when sucrose and the β (1-2) glucan were used in combination, the retention values at 40ºC were as high as those obtained with trehalose at 22ºC (Figure 4). The glucan increased the retention of liposomes preserved with sucrose, and stored at 40ºC for eight

Figure 3. Effect of sucrose/peptone mixture (□;○) and trehalose (■;●) on survival (log of viable cells/mL) of two rhizobial strains freeze-dried, stored over a saturated solution of NaBr and incubated at 4, 30, or 40ºC for 280 hours.
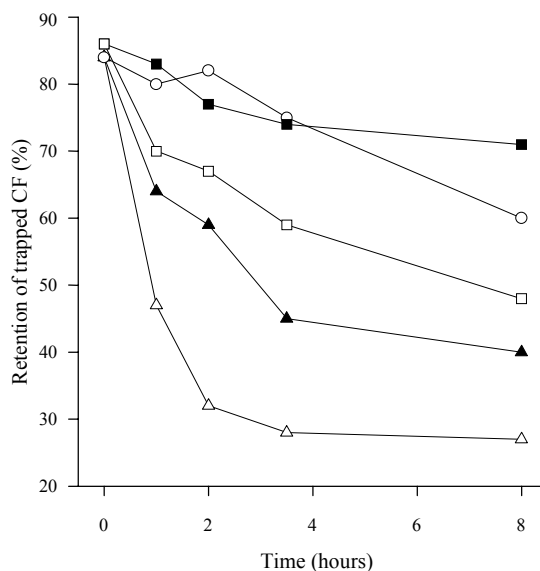

Figure 4. Retention of carboxyfluorescein by egg phosphatidylcholine liposomes after freeze-drying in the presence of various carbohydrates and storage at different temperatures (■:trehalose, 20ºC; ○:sucrose + glucan, 40ºC; □: trehalose, 40ºC; ▲:sucrose, 22ºC; △:sucrose, 40ºC) in a sealed chamber over a saturated NaBr solution. All additives were present at a concentration of 2 g/g lipid before drying.

hours, from 25% to 75%. Therefore, glucan caused a three-fold increase in the level of protection afforded by sucrose, although, the β (1-2) glucan by itself is not at all protective.

The glucan isolated from CIAT 899 cells provided no protection to the lyophilized liposomes (Figure 3). This result was similar to that found for the monosaccharide glucose (Crowe et al., 1997). However when glucose and the glucan were used in combination, there was some protection from damage, as seen by the 44% retention value. This result suggests that glucose and the β (1-2) glucan might contribute differently to membrane protection, and that when both are present in the correct ratio, retention values are increased.

Both trehalose and sucrose alone or in combination with the glucan, provided excellent protection against lyophilization-induced damage (Figure 5). Retention values reached the 80% range in both cases when the disaccharide concentration reached about 10 g additive/g lipid. In fact, in the absence of
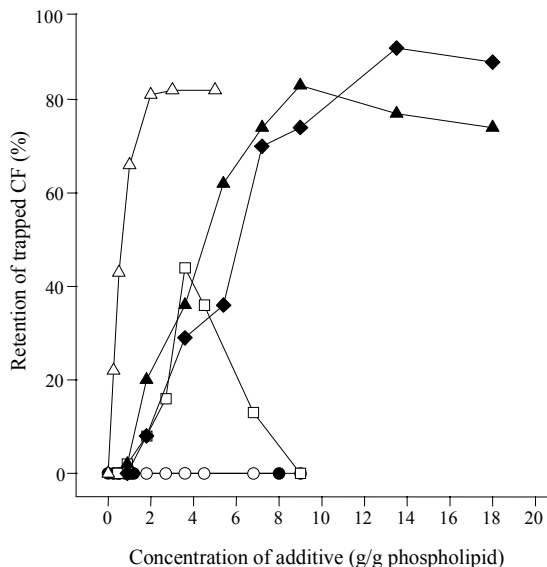
the glucan, liposomes preserved with either trehalose or sucrose can show retention values in the 80% range with as little as 2 g additive/g lipid.

At either extreme of the glucan to glucose spectrum (either 100% glucan or 100% glucose), CF retention was 0% (Figure 6). There was a range in the middle with a peak at a glucose weight fraction of 0.75, in which a significant percentage of carboxyfluorescein was retained by the liposomes (44%). As mentioned before, this finding suggests that glucose and the glucan might each make a specific contribution to membrane stabilization, but that neither one is capable of preserving membrane integrity by itself. Thus, when the glucan is present at high concentrations, glucose cannot gain access to the membrane, and vice versa. The membrane is only protected, therefore, when the two carbohydrates are present in the correct ratio.

In order to determine if the contribution of the β (1-2) glucan to protection of the liposomes and bacteria was due to its formation of a high-melting glass, DSC was used to measure glass transitions in carbohydrate samples of many different hydration levels over a broad temperature range. Most long
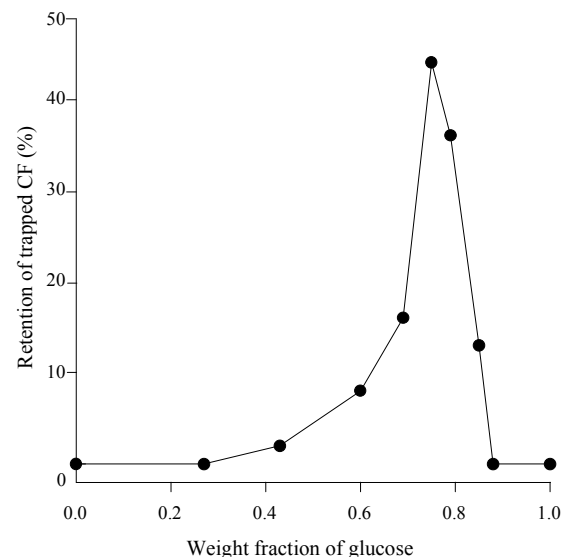

Figure 5. Dose response effect of various sugars (●: glucan; ○: glucose; □: glucose + 1.2 g glucan/g phospholipid; ▲: sucrose + 1.2 g glucan/g phospholipid; ◆: trehalose + 1.2 g glucan/g phospholipid; △: trehalose) on carboxyfluorescein retention by freeze-dried liposomes. Samples were rehydrated and measured immediately after removal from the lyophilizer.

Figure 6. Retention of carboxyfluorescein by liposomes freeze-dried in the presence of various combinations of glucose and β (1-2) glucan (0.0 = 100% of glucan; 1.0 = 100% of glucose).

chain carbohydrates have T_g values of about 200°C under anhydrous conditions (Slade & Levine, 1995). However, in thermal scans between -100 and 150°C, no glass transition was found in dry glucan samples, which decomposed at temperatures below 150°C. Therefore, vitrification is unlikely to be the mechanism by which the glucan protects liposomes and bacteria during freeze-drying. The DSC scans in hydrated glucan samples also gave unexpected results. Figure 5 shows a temperature scan of a hydrated glucan sample between -50 and 100°C. No glass transition is evident, providing further evidence that vitrification of the glucan is not involved in its effects on stability of the rhizobial cells and liposomes in the dry state. However, a thermal event is clearly discernible at 73°C in the fully hydrated glucan. This value is outside a reasonable temperature range for a glass transition in a hydrated sample, even for a polymer (Slade & Levine, 1995). Nevertheless, these high temperature transitions were quite repeatable, so the possibility that they might provide some information about the mechanism of protection was explored.

Another striking result from the DSC work was the amount of water associated with fully hydrated glucan samples. A value of 3.1 g unfrozen water/g dry weight (± 0.4 g/g; n = 4) was calculated, which is several-fold greater than that seen in common carbohydrates. Trehalose and sucrose, for example, have values of only 0.56 and 0.20 g/g, respectively (Levine & Slade, 1988). This ability of the glucan to absorb water was evident in its physical character as well, as it forms a gel in the fully hydrated state.

Thus, it was hypothesized that the thermal events seen in DSC scans of hydrated glucan in the range of 70°C might be gel-sol transitions in this highly hydrated polymer (Figure 7). This possibility was investigated in the following way. A droplet of the hydrated gel was transferred to a 1 mm diameter x 4 cm capillary tube, occupying about 2 mm of the length of the tube. A thermocouple was threaded into the droplet, after which the tube was sealed with Critoseal putty and then heated over a hot water bath. Visual observation of the glucan showed abrupt melting of the gel at 65-75°C in four samples, indicating that the thermal event seen in DSC, also between 65-75°C (0 = 69.6±4.2°C, n = 7), is likely to be this gel-sol transition.
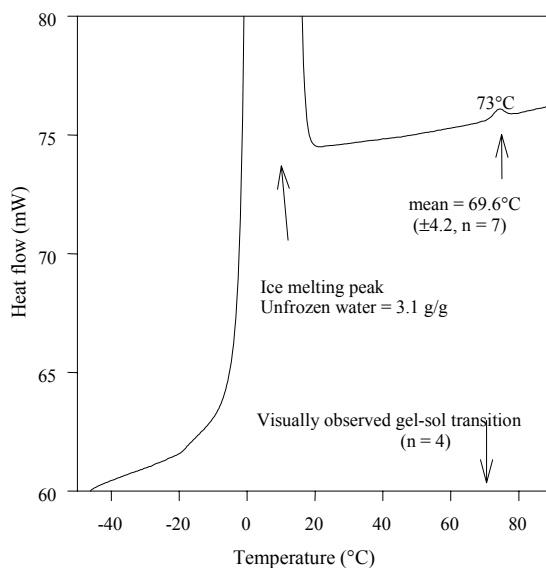

Figure 7. Differential scanning calorimetry spectrum of hydrated β (1-2) glucan. The trace shows a dialyzed glucan sample, in which no glass transition temperature is apparent. The large endotherm, with an onset of 0°C, is the ice-melting peak. The unfrozen water associated with the glucan was calculated from these peaks as 3.1 g water/g dry weight (± 0.4 g/g, n = 4).

The β (1-2) glucan may be instrumental in stabilizing dry samples, therefore, not because of its glass forming properties, but because of its ability to form this hydrated gel. It was hypothesized that the glucan adsorbs water from the vapor phase preferentially, preventing lipids and other carbohydrates present from becoming hydrated. Trehalose is an example of a carbohydrate that can act in this manner. Initially, as moisture is added to dry glasses formed from trehalose and liposomes, a portion of the sugar forms trehalose dihydrate (Crowe et al., 1996). The result is that the water is effectively sequestered away from the glass, and the T_g of the glass remains high (Crowe et al., 1996).

Conclusions

1. CFN 42 cells show a significant decrease in survival when they are lyophilized in sucrose/peptone combination in comparison with cell lyophilized with trehalose.

2. The rhizobial strain CIAT 899 is preserved by sucrose/peptone as well as by trehalose.

3. The β (1-2) glucan abundantly produced by strain CIAT 899 has a strong affinity for water, as evidenced by the large amount of unfrozen water associated with the glucan; this characteristic, which leads to gel formation in fully hydrated samples, may partially explain its protective properties.

Acknowledgements

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